STUDIES IN THE PHYSIOLOGY OF THE FUNGI

XIII. The Effect of Hydrogen-Ion Concentration upon the Accumulation and Activation of Amylase Produced by Certain Fungi

JOANNE L. KARRER

Research Assistant to the Missouri Botanical Garden

Introduction

The effect of the hydrogen- and hydroxyl-ion concentration upon the growth of organisms, in general, has received considerable attention in late years, but the effect of these ions upon the individual processes produced by an organism has not been so thoroughly studied. It has been repeatedly shown that certain fungi require an acid medium to produce maximum growth, while others require alkaline conditions. These diverse relations would seem to indicate a difference in the production, accumulation, and activation of the various enzymes concerned in the growth of these organisms. Recent work has indicated a difference in animal and plant proteolytic enzymes, and it has been shown that plant amylases require a range in conditions different from pancreatic amylase, with regard to activity. In plants, secretion amylase has been found to have properties quite different from those of translocation amylase.

It has been the purpose of this investigation to study and compare the amylases produced by fungi requiring different ranges of H-ion concentration for growth. An attempt has been made to determine the effect of acidity and alkalinity upon the secretion and accumulation of the enzyme. Further, an endeavor has been made to determine whether the enzymes produced under these conditions have similar activities in buffered solutions covering a range of H-ion concentrations, and whether there is any correlation between the optimum for activity and the optimum for secretion and accumulation. It was considered unnecessary to determine the effect of the H ions apart from the other ions in the buffered solution, since the purpose was not to determine the absolute optimum H-ion concentration for the activity of the enzymes but merely to produce a set of similar

conditions under which amyloclastic action could be studied. Thus, a means was furnished for determining whether the amylase produced by organisms requiring different H-ion concentrations for growth would be similar.

Whether the enzymes produced by one fungus or different fungi under widely divergent environmental conditions have similar properties might assist in explaining such problems in parasitism as host specialization and the establishment of strains, and in saprophytism, specialization as to habitat.

SURVEY OF LITERATURE

The influence of acids and alkalis upon the activities of various enzymes was early noticed by different investigators. Pasteur, in 1879, observed the effect of acidity upon the alcoholic fermentation of wines and beers. Probably the first careful work on the influence of small quantities of acids and alkalis upon amyloclastic action was done by Kjeldahl ('79). He showed that small quantities of mineral and organic acids increased the saccharogenic activity of a malt extract, while large amounts caused retardation.

A voluminous literature has been developed, since that time, upon this aspect of amyloclastic activity. The early work relating to the influence of acids and alkalis is very conflicting, both acid and neutral conditions being given as producing optimum activity. Many of these inconsistencies have arisen from a lack of means for determining the exact concentration of H and OH ions in the solution used in the experiment, and thus the data are often difficult to interpret. For this reason, the results of the early investigators have been omitted from the following discussion. An adequate review of the more important literature may be found in the articles by Sherman and his associates ('15) and in the texts by Bayliss ('14), Euler ('12), and Green ('99).

The perfection of methods for measuring H ion concentration, as discussed by Sörensen ('09) and Michaelis ('14), has been a means of obtaining valuable data in regard to the effects of H ions upon enzyme action. The establishment of definite P_H ranges for the indicators has cleared up many discrepancies. Thus, in the work of Maquenne and Roux ('06), the maximum

initial activity of malt amylase was found to be in a solution neutral to methyl orange, but a solution alkaline to this indicator showed maximum total digestion of starch to maltose and glucose. Fernbach and Wolff ('06), working along the same line, found maximum amyloclastic and saccharogenic activities in solutions neutral to methyl orange. A secondary phosphate added to these solutions caused a depression of activity, while a primary phosphate produced either no effect or slight activation. The latter effect was ascribed to a failure to secure complete neutrality in the control solution. An analysis of their conclusions will show that the results are compatible with more recent work, since the range of color change in methyl orange is from about P_H 3 to P_H 5.

Kellerman ('03) studied the effects of various chemical agents upon the activity of Taka diastase by determining the reduction of the solutions with Fehling's solution. He concluded that at a concentration of N/10 all of the inorganic acids employed completely checked the activity. A dilution of N/1000 gave marked acceleration. The results with malt diastase were somewhat different in that acceleration did not occur until an N/5000 dilution was reached. Organic acids gave, in general, results similar to inorganic acids, but malic and acetic acids did not completely check hydrolysis at N/10 dilution. Acetic acid gave no acceleration until a dilution of N/2500 was reached. These differences were not explained by the author, but were, no doubt, due to differences in the ionization of the acids. Without any exception, the alkalis used seemed to be detrimental or slightly so, even up to N/10,000 dilution.

Cole ('03) reached the following conclusion as to the activity of ptyalin: "The hydrolysis of starch is accelerated by the presence in the solution of electro-negative ions (anions) other than OH ions and depressed by electro-positive ions (kations) and by hydroxyl ions." For example, the chlorine ion in HCl is the factor which increases the action, and in low concentration of acid the depression due to the H ions is not sufficient to show itself against the acceleration of the chlorine ions. Although his conclusions seem to be based upon insufficient data in some instances and his interpretations do not altogether agree with recent researches, he demonstrated that a low concentration of

acid increased the action of ptyalin, whereas larger amounts produced inhibition. He also noticed a difference in the accelerating action of acids in the presence of salts, an observation which was earlier reported by Wood ('94).

The effect of the OH-ion concentration in NaOH and Na_2CO_3 solutions upon the saccharogenic power of three different amylases, Taka, saliva, and an extract of swine pancreas, was determined by Quinan ('09). Although no data on the exact OH-ion concentration were presented, the results are worthy of notice in that they show differences in the activities of the amylases employed. Using 100 mgs. of Taka diastase in 100 cc. of a 1 per cent starch solution and allowing the digestion to proceed 18 hours at 36° C., he found the critical hydroxyl-ion concentration, viz., the concentration at which merely a trace of sugar was present, in the solution containing 2 cc. of N/10 NaOH or 6 cc. of N/10 Na_2CO_3. Under the same conditions 1 cc. of saliva acting for 15 hours was found to yield a trace of sugar in solutions containing 1 cc. of N/100 NaOH or 4 cc. of N/100 of Na_2CO_3. Similarly, in 10 hours, 1 cc. of an extract of swine pancreas yielded merely a trace of sugar in solutions containing approximately 3 cc. N/10 NaOH and less than 10 cc. of N/10 Na_2CO_3. The differences in the effects of these alkalis upon the various diastase activities, he stated, were due to differences in dissociation and thus in the amounts of OH ions present.

Although the investigations by Sörensen ('09) and those later by Michaelis ('14) and his associates were not upon amylase specifically, some of the results deserve mention in this connection. Sörensen, studying the factors influencing the activity of catalase and pepsin, concluded that the activity varied according to the actual H-ion concentration and not to the titratable acidity. He also found that there existed one optimum H-ion concentration for each enzyme, regardless of the acid used, and this optimum was dependent upon the time and the temperature at which the enzyme was allowed to act upon the substrate. Thus, after a few minutes in the case of invertase, inversion occurred most rapidly at P_H 3.68 and as the time increased the optimum was shifted toward the neutral point until, in 32 minutes, the optimum was P_H 4.8. He further noted the similarity of the H-ion curve to the temperature curve in enzymatic activity.

Michaelis, publishing with Davidson and later with Peckstein, established definite H-ion concentration optima for various enzymes and also showed that there is an optimum zone rather than a sharply defined point. Michaelis and Peckstein found that ptyalin formed complex combinations with many neutral salts. The affinity for various anions varied greatly, being greatest with the nitrate ion, slightly less with chlorine and bromine, and very little with the sulphate, acetate, and phosphate ions. Each one of these diastase-complexes was found to be a characteristic compound, the fermentative action upon starch being noticeably different and the H-ion concentration optima with regard to activity also varying. The chlorine complex produced the most reactive combination, the nitrate slightly less, and the sulphate, acetate, and phosphate least of all. The H-ion concentrations producing optimum activity were found to be as follows:

Nitrate-diastase	P_H6.9
Chlorine-diastase	P_H6.7
Phosphate-diastase, Sulphate-diastase, Acetate-diastase	P_H6.1–6.2

A series of investigations carried on by Sherman and his associates has been helpful in indicating a field for the study of the properties and conditions for the action of amylases when purified materials are used.

Sherman and Thomas ('15) determined the H-ion concentration most favorable for the activity of malt amylase at 40° C. The weak and strong acids and the acid phosphates of sodium and potassium all showed an optimum activation in solutions having nearly the same actual acidity, this varying from P_H 4.2 to 4.6. With amounts of strong acid above the optimum concentration, the depression of action was greater than with corresponding excesses of weak acids. The activity was determined by the reduction of Fehling's solution and also by the starch iodide method as in Wohlgemuth's modification. The results with these two methods were found to differ. The amyloclastic action, as measured by the Wohlgemuth method, reached an optimum at a concentration of the activating agent (either salt or acid) much below that which gave optimum

saccharogenic action as determined by the reduction of Fehling's solution.

In a later paper by Sherman and Walter ('17), the action of a very concentrated preparation of malt amylase on purified soluble starch was investigated by observing the rate of formation of maltose in neutral solutions without electrolytes and in solutions containing regulated amounts of HCl, H_3PO_4, and KH_2PO_4. When the optimum H-ion concentration, $C_H = 1 \times 10^{-4.4}$, was reached, the action of the enzyme was increased at all stages. This optimum was the same for all of the above electrolytes. The greater the concentration of the enzyme, the less the effect of the electrolyte.

Sherman, Thomas, and Baldwin ('19) recently studied purified amylases obtained from three different sources,in order to determine the optimum H-ion concentration and to establish the "limits of H-ion concentration within which any enzymic activity is shown and the form of the curve representing the activities at all concentrations of H-ions between these limits." Pancreatic and malt amylase and the amylase of *Aspergillus Oryzae* were chosen as representative of the enzyme as it occurs in higher animals, higher plants, and fungi, respectively. The activity of the enzymes was studied in solutions having a range of approximately P_H 2–10. These solutions contained H_3PO_4 and NaH_2PO_4, Na_2HPO_4 and Na_2CO_3, and, in some cases, NaH_2PO_4 or Na_2HPO_4. The action of the enzyme upon the substrate took place at 40° C. and the analyses were made with Fehling's solution. The results showed that pancreatic amylase was active within a range of P_H 4–10, the optimum being at about P_H 7. Malt amylase was active between P_H 2.5–9, with an optimum at P_H 4.4–4.5, and the amylase of *Aspergillus Oryzae* showed activity between P_H 2.6–8, with an optimum at about P_H 4.8. It was thus shown that the amylase of malt and *Aspergillus Oryzae* possessed similar saccharogenic powers in the solutions used in the experiment, while pancreatic amylase not only had a different range in activity but also possessed a higher optimum. The influence of the electrolyte, as distinguished from the H-ion concentration alone, seemed greatest in the case of pancreatic amylase and least in the case of the amylase of *Aspergillus Oryzae*.

A study of the activity of a purified malt amylase in buffered solutions of varying H-ion concentrations was also made by Adler ('16). The enzyme was allowed to act upon the substrate for one hour at 20° C. and the activity measured by the reduction of Fehling's solution and also by the starch iodide reaction. As a result of these investigations, he found that there was an optimum point of P_H 4.9 and that an increase or decrease in the H-ion concentration resulted in a rapid suppression of action. It was also shown that neutral ions were not without effect, although they exerted an influence less than the H ions. Determinations with polarized light of the reducing substances in the solutions gave unsatisfactory results, since the solutions were not always clear. Adler believed that a direct relation between the reduction of Fehling's solution and the amount of polarization did not always exist, thus indicating the possibility of a difference in the effect of the H-ion concentration upon the dextrin production.

In this connection, the work of Chrzaszcz and Joscht ('17) may be mentioned. They concluded that malt amylase was composed of two clearly defined enzymes, a starch-dissolving enzyme, a sugar-producing enzyme, and a starch-dextrin enzyme, which was considered a resultant of the two foregoing enzymes. The iodine reaction corresponded first to the activity of the starch-dissolving and then to the sugar-producing enzyme. However, the correspondence was mostly to the soluble portion. The hydroxides, in general, were found to be unfavorable to the production of sugar in the enzymic reaction, but favorable to the starch-dissolving and dextrin-forming complexes.

Using a modified Lintner method, Falk, McGuire, and Blount ('19) studied some vegetable enzymes in fresh, vacuum-dried and air-dried material in relation to acidity, at 37° C., acting for 2 hours. Well-defined optima were obtained at about P_H 6 with cabbage, carrot, and white turnip juices. With yellow turnip juice, the optimum action extended from P_H 4 to 7. The H-ion concentrations of all the juices from fresh and dehydrated material prepared according to the method described were found to be about P_H 6. Thus, it is seen that the optimum H-ion concentration for the amylases coincided with the natural H-ion concentration of the juices. Dehydration decreased the action in every case.

From the preceding discussion, it is evident that in recent years the effect of the H-ion concentration upon amyloclastic activity has been carefully studied by several investigators. Although the results are not voluminous, they have shown that the reaction of the solution has a marked effect upon enzymic activity. They have shown, further, that amylases from different sources may react differently in this respect.

Scarcely any investigations have been undertaken with a view to determining the influence of H- and OH-ion concentration upon the formation of amylase in the organism. In most of the work on the effect of various conditions upon the secretion of amylase, no mention has been made of the concentration of the H ions coincident with the varying conditions. It is not pertinent to the present discussion to review such works as those by Katz, Dox, Hasselbring, Saito, and others who have studied the relation of organic substances to the production and secretion of amylase. However, it is worthy of note that in many cases neutral, alkaline, and acid substrates were used and that no attempt was made to control the H-ion concentration during the experiment.

The results obtained on the influence of inorganic substances on amyloclastic activity are likewise often difficult to interpret. Robbins ('16), in an extended study of the secretion of amylase by *Penicillium Camembertii*, determined the effect of single salts and the absence of salts in a nutrient solution. The fungus was grown for two weeks at 25° C. in various solutions containing an approximately constant amount of starch. At the end of this time, in the single salt cultures, the mycelium was filtered off, the acidity or alkalinity determined by means of methyl orange and phenolphthalein, and the starch and dextrins undigested in the solution measured by a new method which was based on their insolubility in an acidified aqueous alcoholic solution. The salts in the single salt series, with the exception of the acid phosphates, were all neutral salts, so it was very likely that the H-ion concentration at the beginning of growth was the same, since the highest purity chemicals were employed. The reaction of the solution after the growth of the fungus was found to be alkaline to methyl orange and acid to phenolphthalein, which might indicate a variation from about P_H 4–8

and thus would embody acid, neutral, and alkaline conditions. Under these conditions, he found that potassium salts inhibited digestion more than sodium salts, that potassium and calcium did not seem to be connected with amylase formation, and that there appeared to be an intimate relation between nitrogen and amylase formation. In the nutrient solutions, where salt substitutions were made, the H-ion concentrations, when determined by the method outlined in this paper, were found to vary from P_H 3.3 to 5.4. After the growth of the fungus, the H-ion concentration undoubtedly was shifted, a fact which might have been significant in explaining the results. It is worthy of note that a marked difference was observed by Robbins between the speed with which *Aspergillus Oryzae* and *Penicillium Camembertii*, on the one hand, and *Fusarium* sp. and *Mucor Rouxii*, on the other, digest soluble starch in the absence of all nutrients. The former had a very slow rate while the latter showed fairly rapid digestion.

That invertase formation in yeasts is dependent upon acidity has been shown by Euler and Svanberg ('19). Optimum accumulation was effected at an H-ion concentration of P_H 5–6 and a concentration of P_H 2 was found to be destructive.

Further, Euler and Emberg ('19) attempted to determine the influence of acidity and alkalinity upon enzyme formation by a bottom yeast and the adaptation of these living cells to nutrient solutions. They showed that the maximum H- and OH-ion concentrations at which the cells reproduce themselves or exhibit enzymic relations could be modified by adaptation.

Methods and Materials

Organisms

The fungi used in this investigation were selected with a view to obtaining a group of parasitic organisms which differ in optimum growth with reference to the H-ion concentration of the medium. One organism producing maximum growth in acid media, one growing well in alkaline media, and one requiring either acid or alkaline media were thus chosen. *Colletotrichum Gossypii* (Southworth), *Penicillium italicum*,[1] and *Fusarium* sp.

[1] Determined by Dr. Chas. Thom.

were taken as representative of these conditions. Duggar, Severy, and Schmitz ('17) have shown that *C. Gossypii* is a form which produces growth on media having an alkaline reaction and also that it shifts towards the alkaline side the reaction of certain sugar-containing media upon which it is growing. The fact that *P. italicum* grows abundantly upon citrus fruits whose reaction varies from P_H 2.2 to 4.1 seemed to indicate that it was an organism which could be used to represent activities occurring on the acid side. The culture of *Fusarium* sp. was isolated from cotton and is the same organism as used by Webb ('19). He found that the spores had a wide range for germination in relation to H-ion concentration, varying from P_H 2.8 to 10+ in the NaOH-H_3PO_4-mannite solution used. By employing these forms, it was thought that the amylase formation under different H-ion concentrations could be studied.

The cultures from which spores were obtained for the subsequent inoculations were on media which produced abundant sporulation at room temperature. A synthetic medium prepared according to the following formula[1] was used for *C. Gossypii*.

$MgSO_4$	.25 gms.
K_2HPO_4	.25 gms.
Peptone	10.0 gms.
Glucose	20.0 gms.
Agar	15.0 gms.
Water	1000 cc.

Fusarium sp. was grown on potato agar made according to the method described by Duggar, Severy, and Schmitz ('17), while Czapek's solution containing starch as the source of energy and 1.5 per cent agar was found to produce abundant spores in the case of *P. italicum*.

Spore suspension—The spores used in making the subsequent inoculations for the enzyme studies were from cultures about 14 days old. A uniform suspension was obtained by putting the spores from the culture in 5 cc. of sterile doubly distilled water. A microscopical examination by means of a hanging drop was made of a loopful of this suspension. An average of 6 spores to a field under low power was taken as the standard.

[1] This formula was furnished by Prof. Barre, of Clemson College, S. C. The original citation is unknown to the author.

PREPARATION OF CULTURES FOR ENZYME STUDIES

Chemicals and glassware.—In all of the following experiments the highest purity chemicals and water redistilled from a trace of potassium permanganate and a few drops of sulphuric acid were used unless otherwise specified. The doubly distilled water gave an H-ion concentration of P_H 5.2–5.6. The glassware was chemically cleaned and rinsed with distilled and doubly distilled water except in the experiments for the sugar determinations where distilled water was employed.

The fungi were grown in 300-cc. Pyrex flasks, and the culture solution was prepared according to the following modification of Czapek's solution:

$MgSO_4$	.5 gms.
KH_2PO_4	1.0 gms.
KCl	.5 gms.
$FeSO_4$	.01 gms.
$NaNO_3$	2.0 gms.
Soluble starch	5.0 gms.
Cane sugar	.05 gms.
Water	1000 cc.

The monobasic potassium phosphate was extremely acid and therefore it was recrystallized until a solution of M/15 gave a reaction of P_H 4.5. Starch was added as the source of energy, since Dox ('10) and also later investigators have shown that its presence in culture solutions increases the production of amylase. It has been shown, further, that the presence of a trace of sugar, which increases the initial growth, is beneficial to the formation of amylase.

A range of P_H 1.8–9.4 was obtained in this culture solution by the addition of regulated amounts of N/20 KOH and N/5 HCl according to the method described by Karrer and Webb ('20). From their curve, the amounts of acid and alkali necessary to bring the solution to the required H-ion concentration can be computed. The solutions containing 5 and 10 cc. of N/20 KOH all showed a slight precipitate. Fifty flasks containing a solution of the same H-ion concentration constituted a series. The flasks were inoculated with a loopful of spore suspension, one uninoculated flask being kept as a control. Five series with the culture solution adjusted to P_H 3.0, P_H 4.5, P_H 7.0,

P_H 8.2, and P_H 9.2, respectively, were studied in the case of *Fusarium* sp.; for *Colletotrichum Gossypii*, solutions of P_H 4.5, P_H 7.0, P_H 8.2, and P_H 9.2 were employed; and *Penicillium italicum* was grown in solutions of P_H 3.0 and P_H 4.5. The flasks were placed in the dark at a constant temperature of 28° C. for 2 weeks. At the end of this time, dry weight determinations were made and the final H-ion concentration of the culture media taken. Dry weight determinations were made of the fungous mats in 10 of the flasks. The material was poured upon a weighed filter-paper in a Gooch funnel, thoroughly washed with doubly distilled water, and dried by means of suction. These mats were first allowed to dry in the air for 24 hours and then over $CaCl_2$ in a desiccator for about one week. After this the final weighings were taken. The remaining cultures were filtered and dried in a similar manner.

The H-ion concentration of the control culture solution and of the solution upon which the fungus had been grown was taken according to the method of Clark and Lubs ('17). After 1 per cent toluene was added to the culture solution, it was stored in a refrigerator for subsequent enzyme determinations, which were made within 1–3 days.

ENZYME STUDIES

Amyloclastic activity was studied with the fungous mycelium and with the culture solution.

Preparation of materials.—A series of buffer solutions of different H-ion concentrations in which enzyme activity could be tested were prepared by varying the amounts of N/5 NaOH added to a given volume of H_3PO_4, according to the orthophosphoric acid titration curve given by Clark and Lubs ('17). Solutions of P_H 3, 4, 6, 7, 8, 9, and 11 and doubly distilled water were used in a series. With the exception of the P_H 4 solution, the H-ion concentration coincided with that calculated from the curve. In the case of P_H 4, the critical point as seen in the curve, is so sharply defined that the addition of a small fraction of a cubic centimeter of NaOH resulted in a decided change in the H-ion concentration. Thus, this solution was usually about P_H 5.2.

A 5 per cent soluble starch solution, which was to be added to the above as a substrate, was prepared by mixing the requisite amount of Lintner's soluble starch (Merck) and water and refluxing for about 3 hours. One per cent of toluene was added to the stock solution as a preservative.

Extracellular amylase.—It was found in preliminary experiments that the total volume of the nutrient solution remaining in the various flasks after the growth of the fungus averaged about 40 cc. in all the series, so it was unnecessary to bring the total volume of the solution to a definite volume.

In studying the extracellular amylase, 39 cc. of each of the above-described solutions were placed in 100-cc. Erlenmeyer flasks. To each of these, 1 cc. of the starch paste and 10 cc. of culture solution were added, thus making the total volume 50 cc. One per cent toluene was used as a preservative. The solutions were then incubated at 28° C. for 24 hours. A smaller quantity than 10 cc. of culture solution containing the excreted enzyme was insufficient to produce marked activity in some cases, and a greater amount often caused too much variation in the final H-ion concentration of the solution. Further, an incubation period of 6–12 hours was found to give such low diastatic values that a longer period of incubation was employed. A control series was set up by adding 10 cc. of the culture solution, which had been inactivated by heating in a boiling water-bath for 15 minutes and then made up to the original volume, to solutions of the above H-ion concentrations. In this manner the effect of the H-ion concentration upon the reagents, and also the reducing power of the enzyme solution was determined. A determination was made of the amount of reduction occurring in the enzyme solution alone. This was found not to vary during the course of the experiment.

Method of determining the enzymic action.—At the end of 24 hours, the saccharifying power of the enzyme was tested according to the following method. Ten cc. of each of the buffered solutions and distilled water were accurately pipetted into each of two 50-cc. centrifuge tubes and made neutral to phenolphthalein by adding either NaOH or H_3PO_4. The tubes were then plunged into boiling water for 10 minutes in order to inactivate the enzyme. The same procedure was followed for the inacti-

vated series. Five cc. of Fehling's solution, prepared according to the standard formula, were added to four of these tubes at a time, and the tubes immersed immediately in an actively boiling water bath for 10 minutes. The amount of reduction was determined by the Bertrand method as described by Shaffer ('14), N/50 $KMnO_4$ being used in the titrations. The results were all obtained in duplicate. These differed from each other by not more than .03 cc. of N/50 $KMnO_4$. In all cases the H-ion concentrations of the control and active solutions were taken at the beginning and end of the period of incubation. These were found to remain constant during the time of the experiment. The H-ion concentration was shifted somewhat in several instances after the enzyme dispersion was added, this being due to the presence of alkali or to the presence of salts which affected the original buffer solution.

Intracellular amylase.—The dry fungous mats were powdered in a mortar, and 2.5 gms. were again ground with about an equal amount of powdered Pyrex glass. The enzyme was extracted from this mixture with 125 cc. of doubly distilled water for 12 hours. An extraction of from 1 to 4 hours was found to yield a dispersion of weaker amyloclastic activity. This dispersion was filtered and its activity tested at different H-ion concentrations in a manner similar to that discussed for extracellular amylase, except that 44 cc. of the buffer solution, 5 cc. of enzyme extract, and 1 cc. of 5 per cent soluble starch were used. The enzyme activity was tested exactly as described above.

EXPERIMENTAL DATA AND DISCUSSION

The experimental data will be discussed under the following topics:

(1) An analysis of the cultures, embodying the effect of the H-ion concentration upon the dry weight of the fungus and the effect of the growth of the fungus upon the final H-ion concentration of the culture solution, together with notes on some cultural characteristics of the organisms.

(2) The influence of H-ion concentration of the NaOH-H_3PO_4 solutions upon the activity of the amylase produced in culture solutions having different H-ion concentrations.

(3) The effect of the H-ion concentration of the culture medium upon the accumulation of amylase.

Analysis of the cultures.—From the following table (table 1) it will be seen that the amounts of mycelium of the cultures selected for enzyme determinations when expressed on a dry weight basis were practically the same at the different H-ion concentrations of the nutrient solutions. A small amount of growth is also noticeable in these cases. This, however, was to

TABLE I

DRY WEIGHT DETERMINATIONS OF ORGANISMS EMPLOYED WITH THE INITIAL AND FINAL H-ION CONCENTRATION OF THE CULTURE SOLUTION

Organism	Ser.	Dry wts. in gms.*	Amts. of N/5 HCl and N/20 KOH added in 50 cc. of nutr. sol.		P_H of nutrient solution	
			Cc. N/5 HCl	Cc. N/20 KOH	Initial	Final
Fusarium sp.	I	.0973	0.5	....	3.0	7.2
	II	.0969	....	....	4.5	7.8
	III	.1061	....	5.0	7.0	9.0
	IV	.1010	....	10.0	8.2	9.0
	V	.1023	....	20.0	9.2	9.2
Colletotrichum Gossypii	VI	.1069	....	....	4.5	6.7
	VII	.0976	....	5.0	7.0	7.9
	VIII	.0982	....	10.0	8.2	8.4
	IX	.0817	....	20.0	9.2	8.6
Penicillium italicum	X	.0897	0.5	....	3.0	6.0
	XI	.0909	....	....	4.5	6.3

* The average amount of growth produced in one flask containing 50 cc. of culture solution.

be expected since the amount of mycelium produced by these organisms is never so luxuriant as that produced by *Aspergillus*, *Botrytis*, and other fungi. Dox ('10) has also pointed out that less growth resulted for the organisms used in his experiments in a nutrient solution containing starch than in one containing some other carbohydrate. With all the cultures of *Fusarium* sp., growth appeared at the end of about 4 days. The mycelium grew on the surface of the solution in a fluffy mat and the spores were produced at all H-ion concentrations. At a concentration

of P_H 2.0 no growth occurred, and the limit on the alkaline side was found to be beyond P_H 9.2. Thus, the growth range followed the range for spore germination as determined by Webb ('19), this being from P_H 2.8 to 10.0+.

With the exception of the P_H 9.2 culture solution, the reaction of all the culture solutions, with starch as the source of energy, was changed during the growth of the fungus, this shift being toward increased alkalinity. The greater the acidity the greater the change produced. Thus, the reaction of the P_H 3.0 culture solutions was shifted to 7.0 while that of P_H 8.2 was shifted to 9.0.

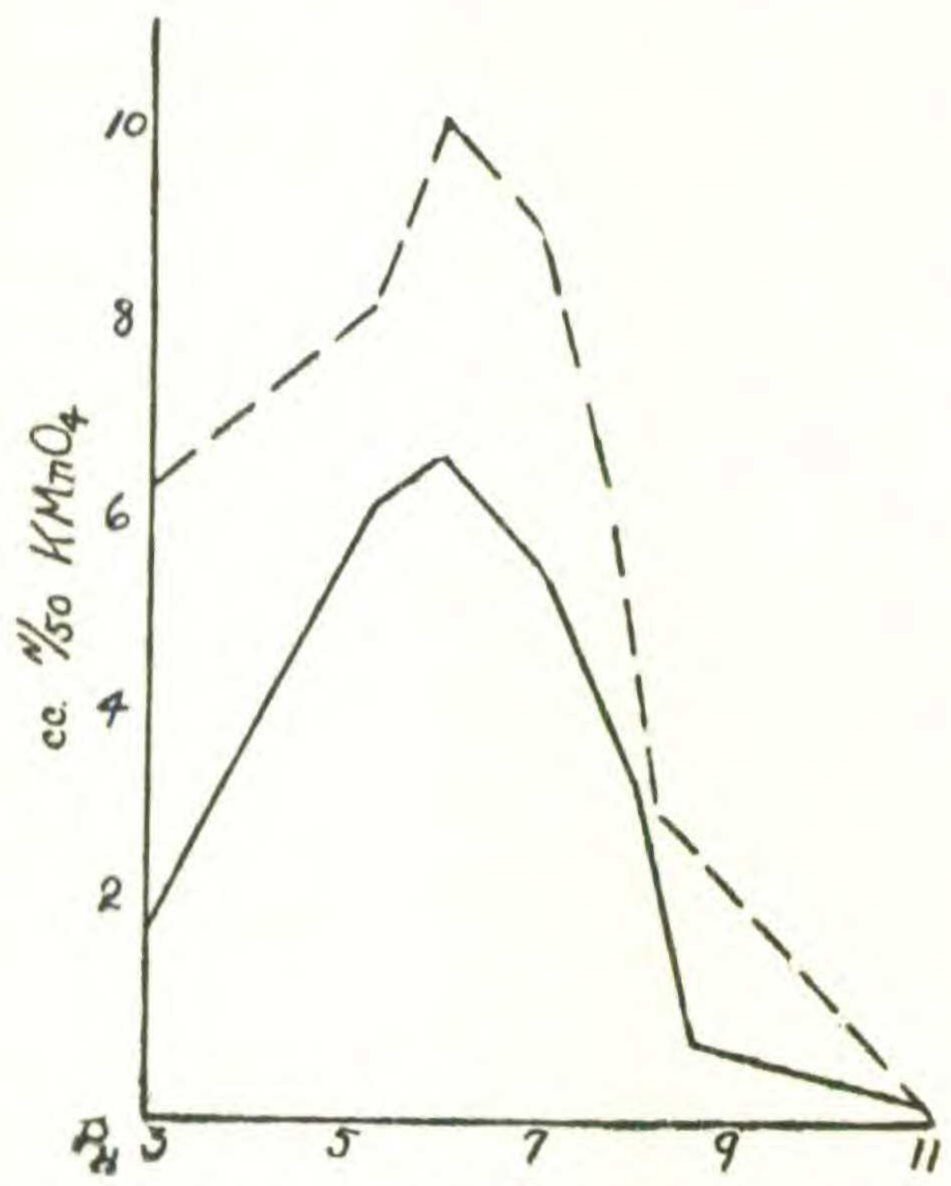

Fig. 1. Action of extracellular (——) and intracellular (- - -) amylase produced by *Fusarium* sp. grown in Czapek's solution of P_H 3.0.

Colletotrichum Gossypii produced visible growth at the end of 3 to 4 days at favorable H-ion concentrations. Abundant spores were produced in the cultures, and the mycelium from the first was very gelatinous and attached to the bottom of the flask. The growth range was found to be from P_H 3.0-4.5 to 9.2 or beyond for the production of a fair quantity of mycelium. The final H-ion concentration of the solution, although shifted toward the alkaline side in all but one instance, gave results, in the corresponding solutions, much less than those of *Fusarium*. The greatest shift was from P_H 4.5 to 6.7 in natural Czapek's solution. The alkalinity of the culture solution with an initial reaction of P_H 9.2 was somewhat lessened during the growth of the fungus, since the final reaction was P_H 8.6.

Penicillium italicum was found to have a more limited growth range with respect to the reaction of the culture solution than

either of the other organisms. The best results were obtained under acid conditions from P_H 2.5 to 4.5. At P_H 8.0 only a few hyphae were produced from the spores. Visible growth appeared at the end of about 4 days, and the mycelium grew on the surface of the liquid forming a rather thin, felt-like mass. No spores were produced in the culture solution having a concentration of P_H 3.0, but there was abundant production in most of the natural Czapek's solution cultures of P_H 4.5.

Amylase activities.—As stated above, the results of the experiments on amylase activity will be discussed from two standpoints: the effect of the buffer solution of various H-ion concentrations upon the activity of the amylase produced in the different culture solutions, and the effect of the H-ion concentration of the culture medium upon its accumulation in the mycelium and the culture solution. The data will be presented in the form of tables, also of curves where the abscissae are the H-ion concentrations of the buffered solutions in which enzyme activity was measured, and the ordinates, the cubic centimeters of the N/50 $KMnO_4$ solution which represent the relative amounts of starch hydrolyzed and therefore the extent of enzyme production. As shown in tables II–IV, the results of the inactivated or control series were subtracted from the active series in order to produce these amounts. The extracellular amylase curve denotes hydrolysis with 2 cc. of culture solution and the curve of intracellular amylase with 1 cc. of 2 per cent mycelium dispersion or .02 gm. of the powdered mycelium.

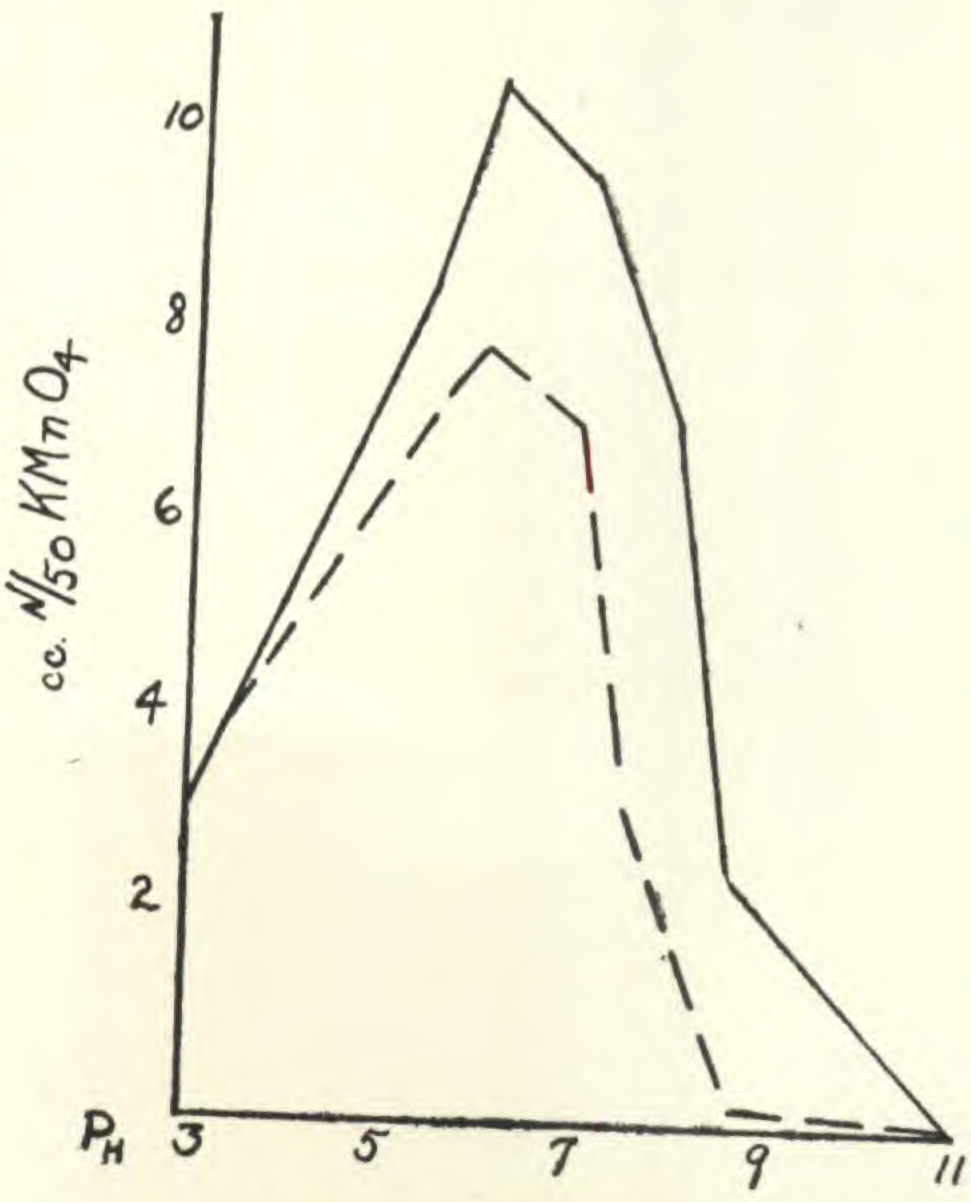

Fig. 2. Action of extracellular (——) and intracellular (- - -) amylase produced by *Fusarium* sp. grown in Czapek's solution of P_H 4.5.

Since the dry weight determinations of Series 1–11 inclusive were equal within the limits of experimental error and the total amounts of culture solution remaining in the flasks after the

TABLE II

SHOWING THE ACTIVITY OF INTRA- AND EXTRACELLULAR AMYLASE PRODUCED BY FUSARIUM SP. WHEN GROWN IN CZAPEK'S SOLUTION HAVING INITIAL H-ION CONCENTRATIONS OF P_H 3.0 AND 4.5

	Initial H-ion concentration of Czapek's solution							
	P_H 3.0				P_H 4.5			
	P_H of buffer sol.*	Cc. N/50 $KMnO_4$			P_H of buffer sol.*	Cc. N/50 $KMnO_4$		
		Total	Control	Amylase activity		Total	Control	Amylase activity
Extracellular amylase	3.0	2.2	0.4	1.8	3.0	4.3	1.3	3.0
	5.3	6.7	0.5	6.2	5.2	9.8	1.3	8.5
	6.0	7.1	0.4	6.7	6.0	12.2	1.4	10.8
	7.0	6.0	0.4	5.6	7.0	11.0	1.3	9.7
	8.0	4.0	0.6	3.4	8.0	8.5	1.5	7.0
	8.6	1.3	0.5	0.8	8.6	4.1	1.5	2.6
	11.0	0.7	0.5	0.2	11.0	1.5	1.6	0.0
	7.2†	6.5	0.5	6.0	7.2†	10.5	1.5	9.0
Intracellular amylase	3.0	9.2	2.8	6.4	3.2	6.6	2.6	4.0
	5.6	11.1	2.8	8.3	5.2	10.1	2.9	7.2
	6.0	13.2	3.0	10.2	6.0	10.8	2.9	7.9
	7.0	11.8	2.8	9.0	6.9	10.1	2.9	7.2
	7.7	9.2	2.8	6.4	7.5	6.4	2.9	3.5
	8.2	6.1	2.9	3.2	8.7	2.8	2.6	0.2
	11.0	3.0	2.8	0.2	11.0	2.6	2.6	0.0
	6.0†	13.3	2.8	10.5	6.1†	9.9	2.5	7.4

* H-ion concentration of the buffer solution in which enzyme activity was measured.

† Doubly distilled water was substituted for the buffer solution.

growth of the fungus were the same, the tables and curves can be compared directly. The amounts of enzyme produced in the culture solution according to the tables will thus represent

TABLE III

SHOWING THE ACTIVITY OF INTRA- AND EXTRACELLULAR AMYLASE PRODUCED BY FUSARIUM SP. WHEN GROWN IN CZAPEK'S SOLUTION WITH H-ION CONCENTRATIONS OF P_H 7.0, 8.2, AND 9.2

	Initial H-ion concentration of Czapek's solution											
	P_H 7.0				P_H 8.2				P_H 9.2			
	P_H of buffer sol.*	Cc. N/50 $KMnO_4$			P_H of buffer sol.*	Cc. N/50 $KMnO_4$			P_H of buffer sol.*	Cc. N/50 $KMnO_4$		
		Total	Control	Amylase activity		Total	Control	Amylase activity		Total	Control	Amylase activity
Extracellular amylase	3.0	0.6	0.6	0.0	3.2	1.1	0.8	0.3	3.0	0.8	0.8	0.0
	5.0	1.6	0.6	1.0	5.7	2.6	0.9	1.7	5.2	2.4	0.7	1.7
	6.8	2.1	0.6	1.5	6.2	2.9	0.9	2.0	6.0	2.5	0.9	1.6
	7.1	1.4	0.5	0.9	7.0	1.9	0.8	1.1	7.0	2.2	0.8	1.4
	8.0	0.6	0.6	0.0	8.0	1.1	0.9	0.2	8.0	1.6	0.8	0.8
	9.0	0.6	0.6	0.0	9.0	0.9	0.9	0.0	9.0	0.8	0.8	0.0
	11.0	0.6	0.6	0.0	11.0	0.9	0.9	0.0	11.0	0.8	0.8	0.0
	7.6†	1.6	0.6	1.0	7.8†	1.3	0.8	0.5	8.0†	1.6	0.8	0.8
Intracellular amylase	3.2	4.4	3.4	1.0	3.1	4.8	3.5	1.3	3.0	4.7	2.4	2.3
	4.4	9.5	3.6	5.9	4.4	5.4	3.5	1.9	5.5	6.3	2.6	3.7
	6.0	9.6	3.7	5.9	6.1	7.3	3.4	3.9	6.0	6.8	2.5	4.3
	6.9	7.8	3.4	4.4	7.0	5.4	3.4	2.0	7.0	5.5	2.3	3.2
	7.6	5.7	3.5	2.2	8.0	3.6	3.4	0.2	8.0	4.7	2.5	2.2
	9.0	3.5	3.5	0.0	9.0	3.5	3.5	0.0	8.3	3.7	2.5	1.2
	11.0	3.5	3.5	0.0	11.0	3.5	3.5	0.0	11.0	2.5	2.5	0.0
	6.4†	8.5	3.5	5.0	6.6†	6.9	3.4	3.5	6.7†	5.6	2.6	3.0

* H-ion concentration of the buffer solution in which enzyme activity was measured.

† Doubly distilled water was substituted for the buffer solution.

TABLE IV

SHOWING THE ACTIVITY OF INTRA- AND EXTRACELLULAR AMYLASE PRODUCED BY COLLETOTRICHUM GOSSYPII WHEN GROWN IN CZAPEK'S SOLUTION HAVING INITIAL H-ION CONCENTRATIONS OF PH 4.5, 7.0, 8.2, AND 9.2

	Initial H-ion concentration of Czapek's solution							
	PH 4.5				PH 7.0			
	PH of buffer sol.*	Cc. N/50 $KMnO_4$			PH of buffer sol.*	Cc. N/50 $KMnO_4$		
		Total	Control	Amylase activity		Total	Control	Amylase activity
Extracellular amylase	3.0	7.1	0.5	6.6	3.0	14.7	0.9	13.8
	5.2	9.3	0.5	8.8	5.1	14.9	0.7	14.2
	6.0	9.4	0.4	9.0	5.9	15.7	0.8	14.9
	7.0	9.2	0.4	8.8	7.0	13.8	0.8	13.0
	8.0	5.9	0.4	5.5	8.0	12.4	0.8	11.6
	8.4	2.5	0.4	2.1	8.5	1.4	0.9	0.5
	11.0	0.5	0.5	0.0	11.0	0.9	0.9	0.0
	6.6†	10.0	0.5	9.5	6.8†	13.7	0.7	13.0
Intracellular amylase	3.0	5.8	0.8	5.0	3.0	13.2	0.4	12.8
	5.2	9.5	0.7	8.8	5.5	14.0	0.4	13.6
	6.1	10.9	0.6	10.3	6.0	15.5	0.4	15.1
	7.0	9.8	0.8	9.0	7.0	13.8	0.5	13.3
	8.2	5.2	0.8	4.4	8.0	12.0	0.4	11.6
	8.4	2.8	0.7	2.1	8.8	7.2	0.5	6.7
	11.0	0.8	0.8	0.0	11.0	0.5	0.5	0.0
	6.0†	11.8	0.8	11.0	6.0†	15.6	0.5	15.1
	PH 8.2				PH 9.2			
Extracellular amylase	3.0	12.1	0.9	11.2	4.4	12.0	1.7	10.3
	5.6	14.2	1.0	13.2	6.2	12.9	1.7	11.2
	6.0	17.2	0.8	16.4	6.5	10.8	1.7	9.1
	7.0	12.6	0.8	11.8	7.2	8.4	1.7	6.7
	8.0	8.4	0.9	7.5	8.1	4.1	1.7	2.4
	9.0	4.1	0.9	3.2	9.1	2.4	1.7	0.7
	11.0	1.0	1.0	0.0	11.0	1.7	1.7	0.0
	7.2†	0.9	0.9	9.0	7.9†	4.6	1.6	3.0
Intracellular amylase	3.0	12.5	1.8	10.7	3.0	15.8	3.6	12.2
	5.2	14.2	1.9	12.3	5.4	18.4	3.7	14.7
	5.9	14.1	1.8	12.3	6.0	18.6	3.6	15.0
	6.9	11.4	1.5	9.9	6.9	15.8	3.8	12.0
	8.0	11.0	1.7	9.3	8.0	11.3	3.6	7.7
	9.0	7.6	1.8	5.8	8.4	7.1	3.6	3.5
	11.0	2.2	1.8	0.4	11.0	3.6	3.6	0.0
	6.4†	12.4	1.8	10.6	7.0†	17.2	3.6	13.6

* H-ion concentration of the buffer solution in which enzyme activity was measured.

† Doubly distilled water was substituted for the buffer solution.

1/20 of the amylase excreted into the culture medium by one dry weight of the fungus. The amounts obtained with intracellular amylase will represent an average of about 1/5 of the total found in the mycelium of one culture.

TABLE V

SHOWING THE ACTIVITY OF INTRA- AND EXTRACELLULAR AMYLASE PRODUCED BY PENICILLIUM ITALICUM WHEN GROWN IN CZAPEK'S SOLUTION WITH H-ION CONCENTRATIONS OF P_H 3.0 AND 4.5

	Initial H-ion concentration of Czapek's solution							
	P_H 3.0				P_H 4.5			
	P_H of buffer sol.*	Cc. N/50 $KMnO_4$			P_H of buffer sol.*	Cc. N/50 $KMnO_4$		
		Total	Control	Amylase activity		Total	Control	Amylase activity
Extracellular amylase	3.0	4.5	1.5	3.0	3.0	5.6	0.4	5.2
	5.2	4.8	1.2	3.6	5.4	5.7	0.4	5.3
	6.0	4.9	1.5	3.4	6.0	6.0	0.4	5.6
	7.0	1.7	1.3	0.4	7.1	2.5	0.5	2.0
	8.0	1.5	1.5	0.0	8.0	0.4	0.4	0.0
	9.0	1.5	1.5	0.0	9.0	0.4	0.4	0.0
	11.0	1.5	1.4	0.0	11.0	0.4	0.4	0.0
	5.7†	5.8	1.5	4.3	6.4†	6.4	0.4	6.0
Intracellular amylase	3.0	15.7	3.7	12.0	3.0	12.7	2.5	10.2
	5.1	16.3	3.7	12.6	5.1	12.2	2.4	9.8
	6.0	16.0	3.5	12.5	6.1	12.5	2.4	10.1
	7.0	9.0	3.6	5.4	7.0	6.7	2.5	4.2
	8.0	3.8	3.6	0.2	8.0	2.5	2.5	0.0
	9.0	3.6	3.6	0.0	9.0	2.5	2.5	0.0
	11.0	3.6	3.6	0.0	11.0	2.5	2.5	0.0
	6.4†	16.6	3.6	13.0	6.4†	10.8	2.5	8.3

* H-ion concentration of the buffer solution in which enzyme activity was measured.

† Distilled water was substituted for the buffer solution.

It will be seen from the curves (figs. 1–5) and tables II and III, representing the enzyme activity of *Fusarium*, that the effect of the buffer solution of various H-ion concentrations upon the extra-and intracellular amylase, produced in culture solutions of the same and of different H-ion concentrations, was similar. The ranges for activity did not correspond in all cases, but this might have been due to the small amount of enzyme material present which at even the optimum H-ion concentration hydrolyzed only a small quantity of starch. It is also likely that under such low enzymic activity the differences in the original enzyme dispersion, which was the culture solution in one case and an extract of the mycelium in the other, might have been a factor. The amount of starch hydrolyzed at P_H 3.0 varied, but a maximum was reached at P_H 6.0 in all of the series. As the buffer solution approached alkalinity, enzyme activity decreased until finally at P_H 11.0 there was complete inhibition. A rather uniform fall in the activity occurred from P_H 6.0 to P_H 11.0.

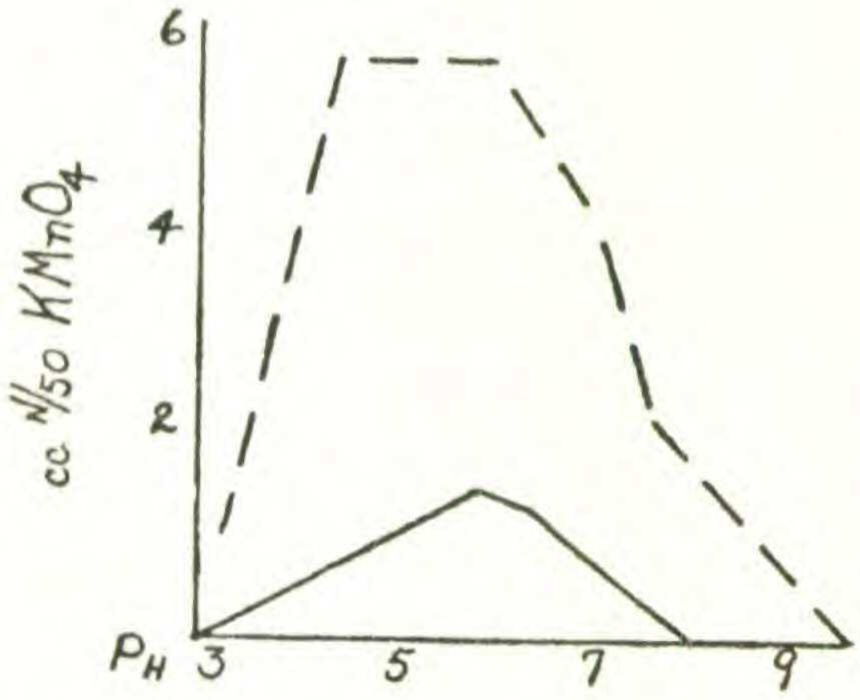

Fig. 3. Action of extracellular (——) and intracellular (- - -) amylase produced by *Fusarium* sp. grown in Czapek's solution of P_H 7.0.

A similarity will also be noticed for the activity of extra- and intracellular amylase produced by *Colletotrichum* (figs. 6–9 and table IV) at various H-ion concentrations. Inhibition by acid occurred below P_H 3.0 and maximum activity was reached at P_H 6.0, beyond which there was a decrease, and at P_H 11.0 the enzyme was completely inhibited.

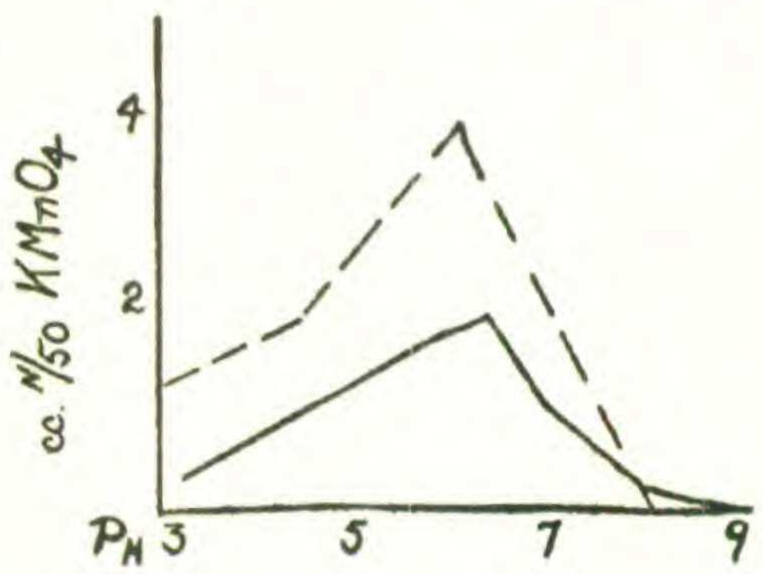

Fig. 4. Action of extracellular (——) and intracellular (- - -) amylase produced by *Fusarium* sp. grown in Czapek's solution of P_H 8.2.

From the two series of cultures of *Penicillium* (figs. 10–11 and table v), it is evident that the effect of the buffer solution upon amyloclastic activity of the intra and extracellular enzyme in general produced the same results. The range of activity extended from below P_H 3.0 to P_H 8.0 where complete inhibition occurred. The amylase as produced by this organism did not have a sharply defined optimum acidity. Any H-ion concentration between P_H 3.0 and 6.0 seemed to be equally favorable for both the extracellular and intracellular enzyme action.

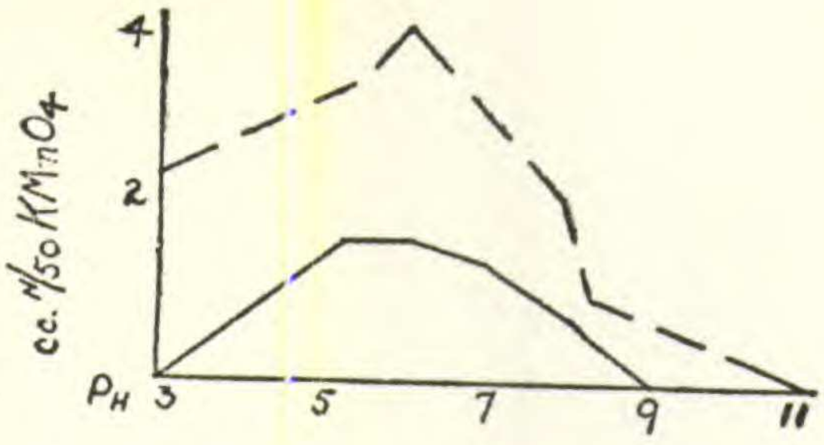

Fig. 5. Action of extracellular (——) and intracellular (- - -) amylase produced by *Fusarium* sp. grown in Czapek's solution of P_H 9.2.

It is worthy of note that when the activity of amylase in all of the series studied was measured in distilled water instead of the buffer solution (tables II–IV), the results obtained agreed closely with those of the corresponding H-ion concentrations of the buffer solution, thus indicating that at these reactions the ions other than the H ions in the buffer solution had little effect. The distilled water used had an H-ion concentration of P_H 5.4–5.8, but upon the addition of the enzyme dispersion this was shifted toward alkalinity.

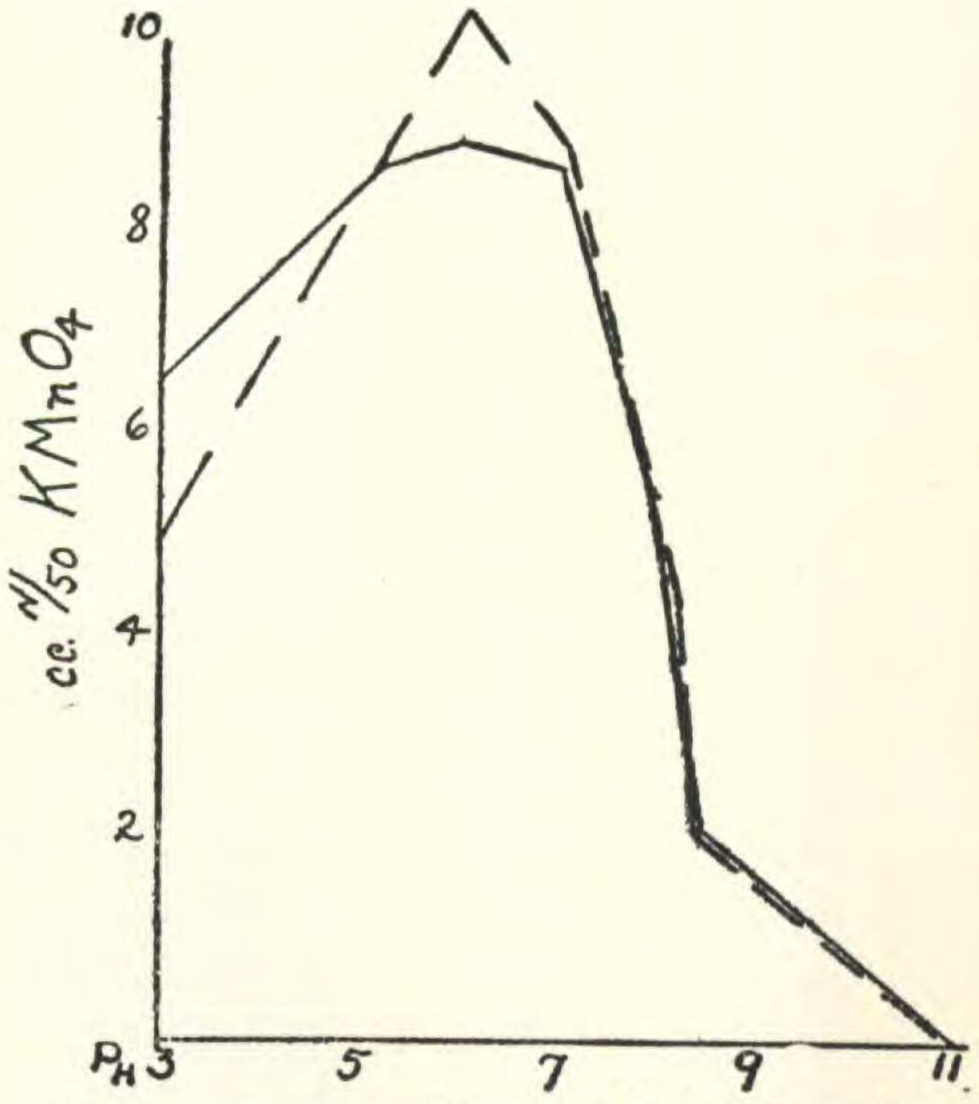

Fig. 6. Action of extracellular (——) and intracellular (- - -) amylase produced by *Colletotrichum Gossypii* grown in Czapek's solution of P_H 4.5.

When the influences of the H-ion concentration upon the enzyme secreted by the same fungus grown in nutrient solutions containing varying amounts of acidity and alkalinity are com-

pared, it is evident that the reaction of the medium seemed to have no influence upon the resulting properties as determined by the buffer solution. It must be remembered, however, that the H-ion concentration of the nutrient solution had, in most of the series, been changed during the growth of the fungus. The greatest difference, as seen in table 1, was in Series 1 and 5 with *Fusarium* where the final reaction was P_H 7.2 and 9.2 respectively. This variation might not have been decided enough to produce a change in the processes within the fungus. The results might have been very different if it had been possible to keep the reaction constant. However, although the P_H 9.2 series of *Fusarium* did not change during the experiment and the more alkaline cultures of *Colletotrichum* were shifted very slightly, the enzyme produced under these conditions was similar to the one produced in the cultures which at the beginning were P_H 3.0 or P_H 4.5. Further, it is impossible to say what the reaction within the cell of the fungus has been during the secretion, but it is significant that the enzyme which had been excreted into the culture solution retained the properties of the enzyme in the mycelium. Again, Euler and Emberg ('19) have shown that the enzyme formation by yeast cells could be modified by the adaptation of the

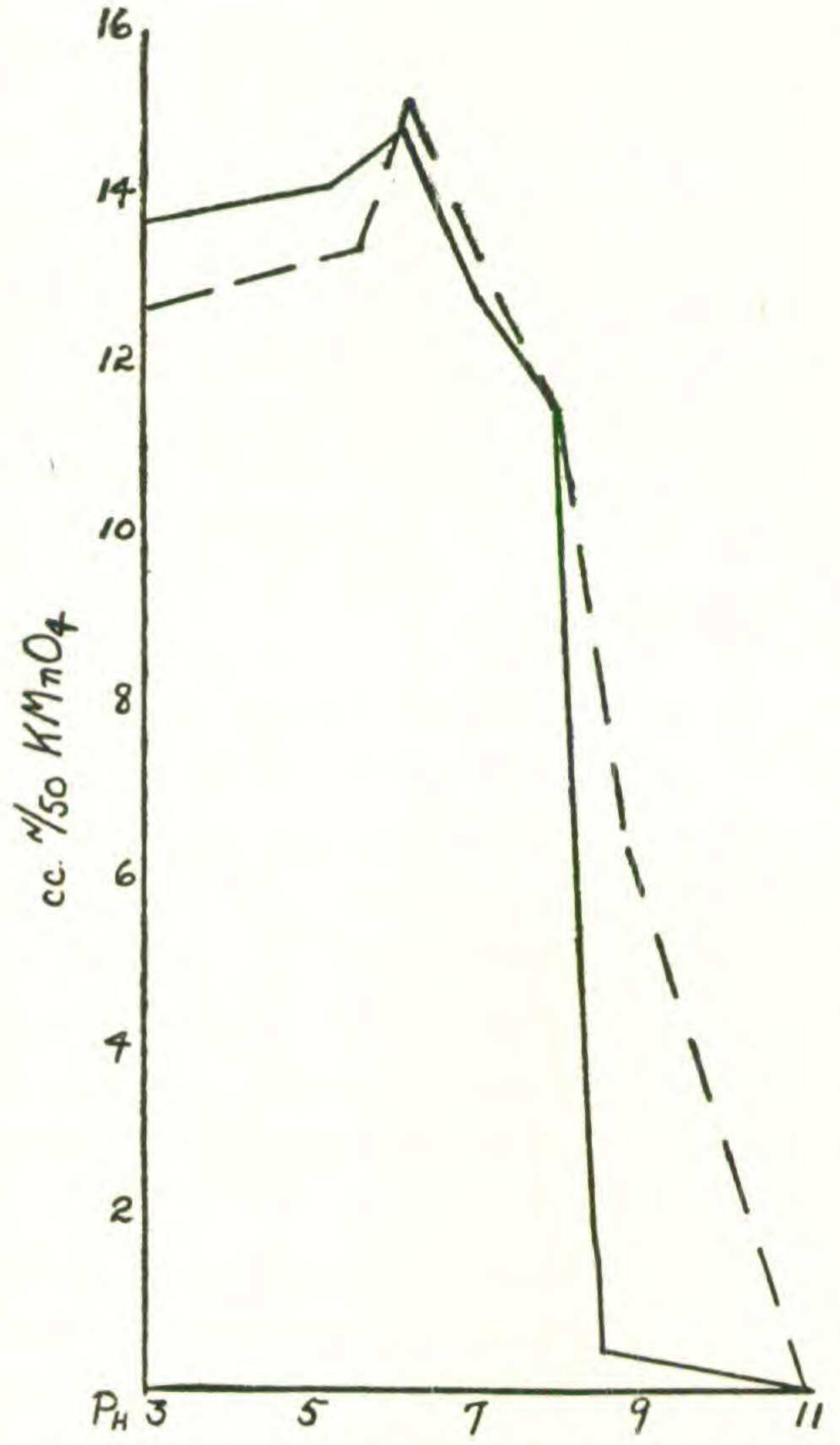

Fig. 7. Action of extracellular (——) and intracellular (- - -) amylase produced by *Colletotrichum Gossypii* grown in Czapek's solution of P_H7.0.

cells to nutrient solutions. If it had been possible to grow the various organisms used in this investigation for several generations upon media having extreme H-ion concentrations with relation to growth more striking results might have been obtained.

Differences with respect to activity range in the buffer solution will be noted in a comparison of the curves of the various organisms. *Fusarium* and *Colletotrichum* resembled each other, while *Penicillium* possessed characteristics somewhat unlike either of these. In the former, maximum activity occurred at P_H 6.0, a gradual decrease followed as the solutions became more alkaline, and complete inhibition occurred at P_H 9.0 or 11.0, depending upon the amount of amylase present in the original enzyme dispersion. In the latter, on the other hand, a zone of maximum action occurred between P_H 3.0, or lower, to P_H 6.0, and activity definitely ceased at P_H 8.0. Even though the enzymes were not purified,

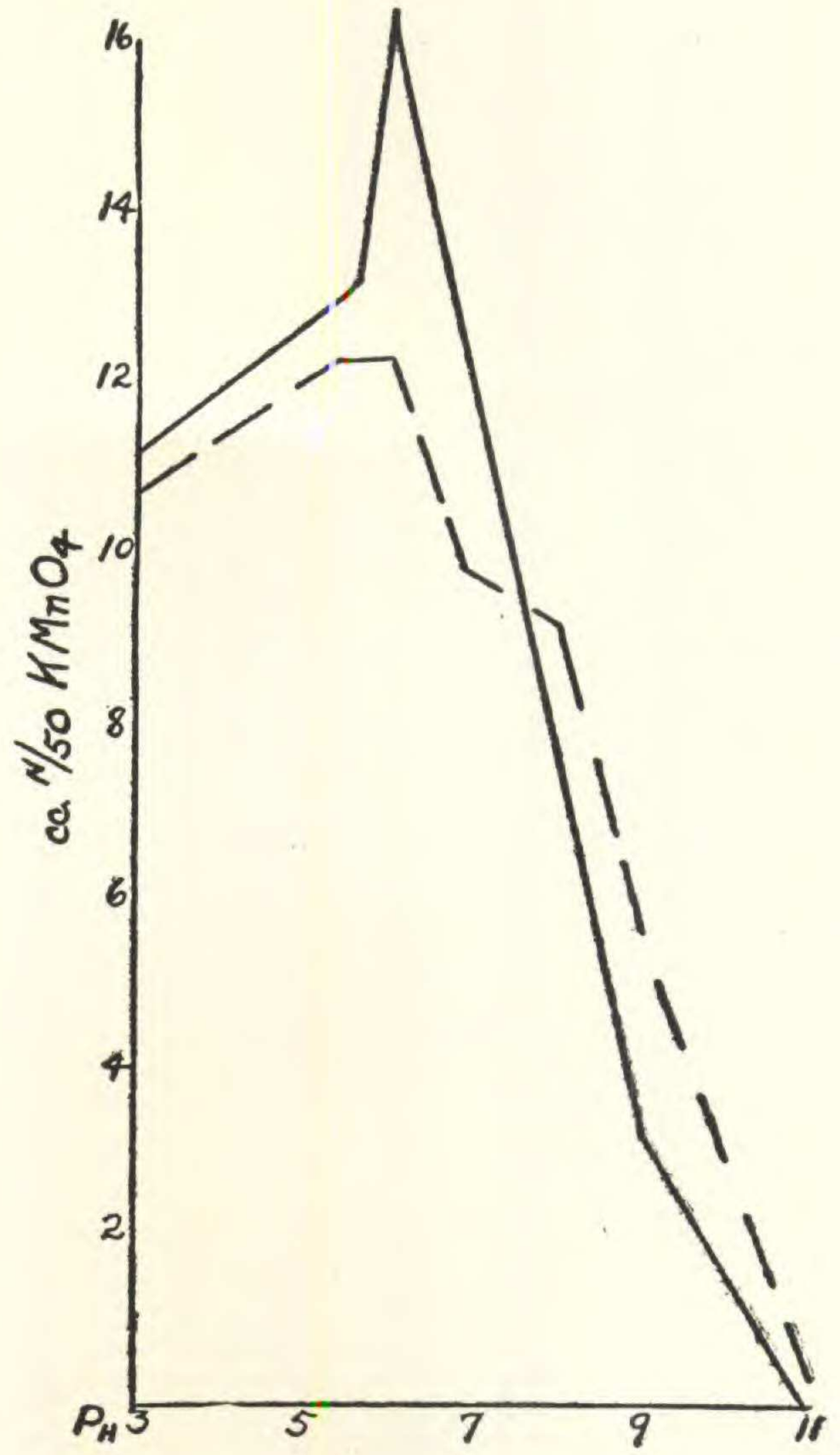

Fig. 8. Action of extracellular (——) and intracellular (- - -) amylase produced by *Colletotrichum Gossypii* grown in Czapek's solution of P_H 8.2.

the results would seem to indicate that in *Penicillium* an amylase was formed which had properties somewhat different, at least in regard to activation by the H_3PO_4-NaOH-starch buffer solution employed for measuring amylase activity.

It was not the purpose of this investigation to establish definite maxima for the amylases produced by these organisms,

still it may be noted that the maximum activity relations as determined by these experiments were different from those obtained by Sherman and his associates ('15–'17) and by Adler ('16). This may be due to the fact that the ions other than the H ions in the solutions used to determine activity exerted an influence. It is very likely, on the other hand, that the time and temperature of incubation had some influence, since Sörensen showed that the H-ion concentration at which maximum activity was produced, in the case of invertase, depended upon the two above factors and the shift seemed to be toward neutrality with continued incubation.

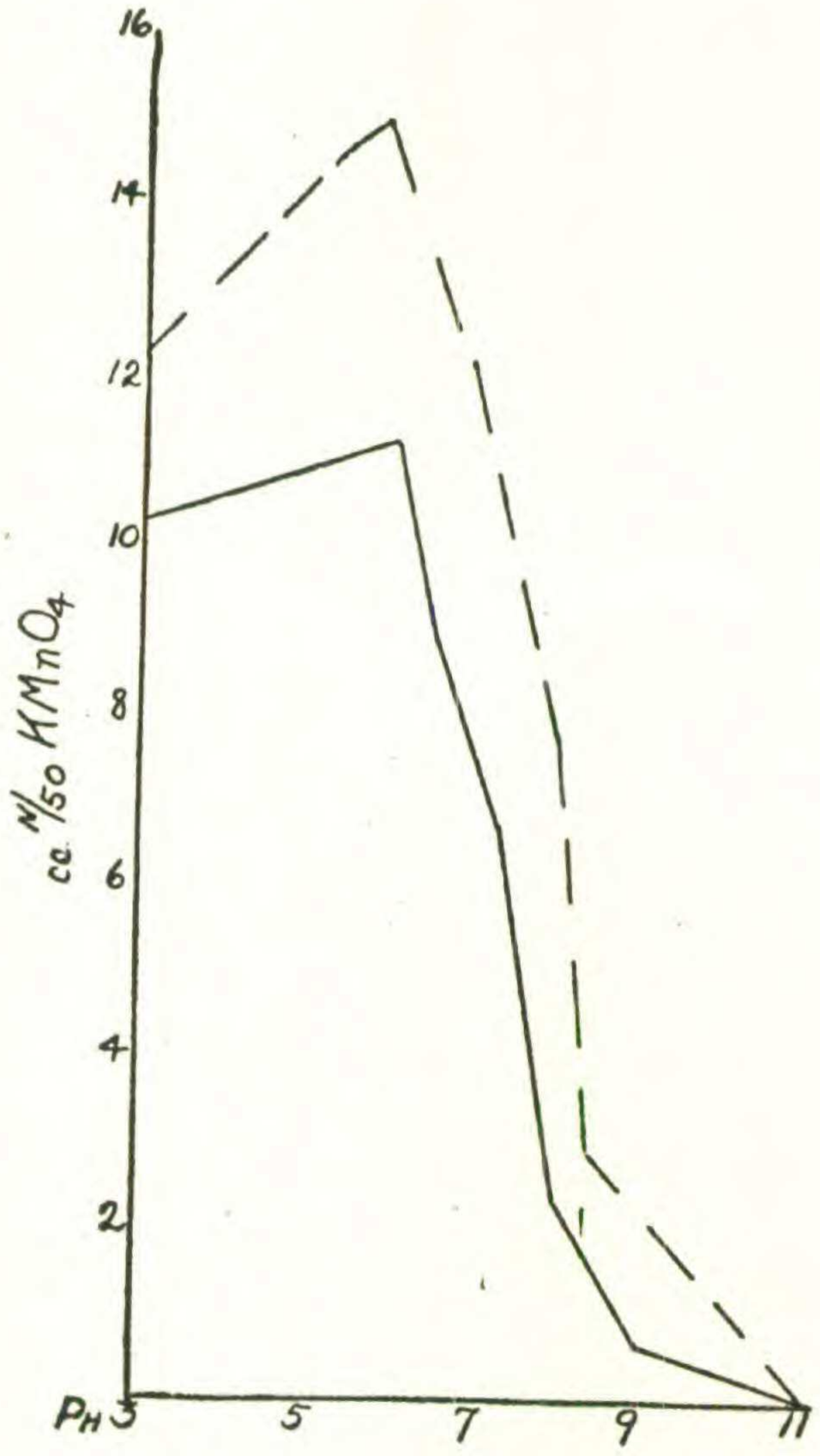

Fig. 9. Action of extracellular (——) and intracellular (- - -) amylase produced by *Colletotrichum Gossypii* grown in Czapek's solution of P_H9.2.

Since the amounts of the culture solution remaining in the flasks after growth of the fungus were the same in all cases and the dry weights varied very slightly, a summation of the activities of the intra- and extracellular enzymes can be taken to denote relative enzyme accumulation. A relation which varied with the organism seemed to exist between the H-ion concentration of the medium and the accumulation of the enzyme. If the maximum activity, which is about P_H 6.0 in the foregoing series, is taken as an index of the amount of amylase present, excretion into the culture solution was greatest for *Fusarium* in natural Czapek's solution, for *Penicillium* in the culture

solution having an initial of P_H 3.0 and for *Colletotrichum* in a medium of P_H 8.2. Intracellular amylase accumulated most abundantly in culture solutions of P_H 3.0 and 8.2 in the cases of *Fusarium* and *Colletotrichum* respectively, while natural Czapek's solution was most beneficial in the case of *Penicillium*.

With *Fusarium*, the greatest amount of total amylase accumulation was in Czapek's solution (fig. 12 and table VI). There was

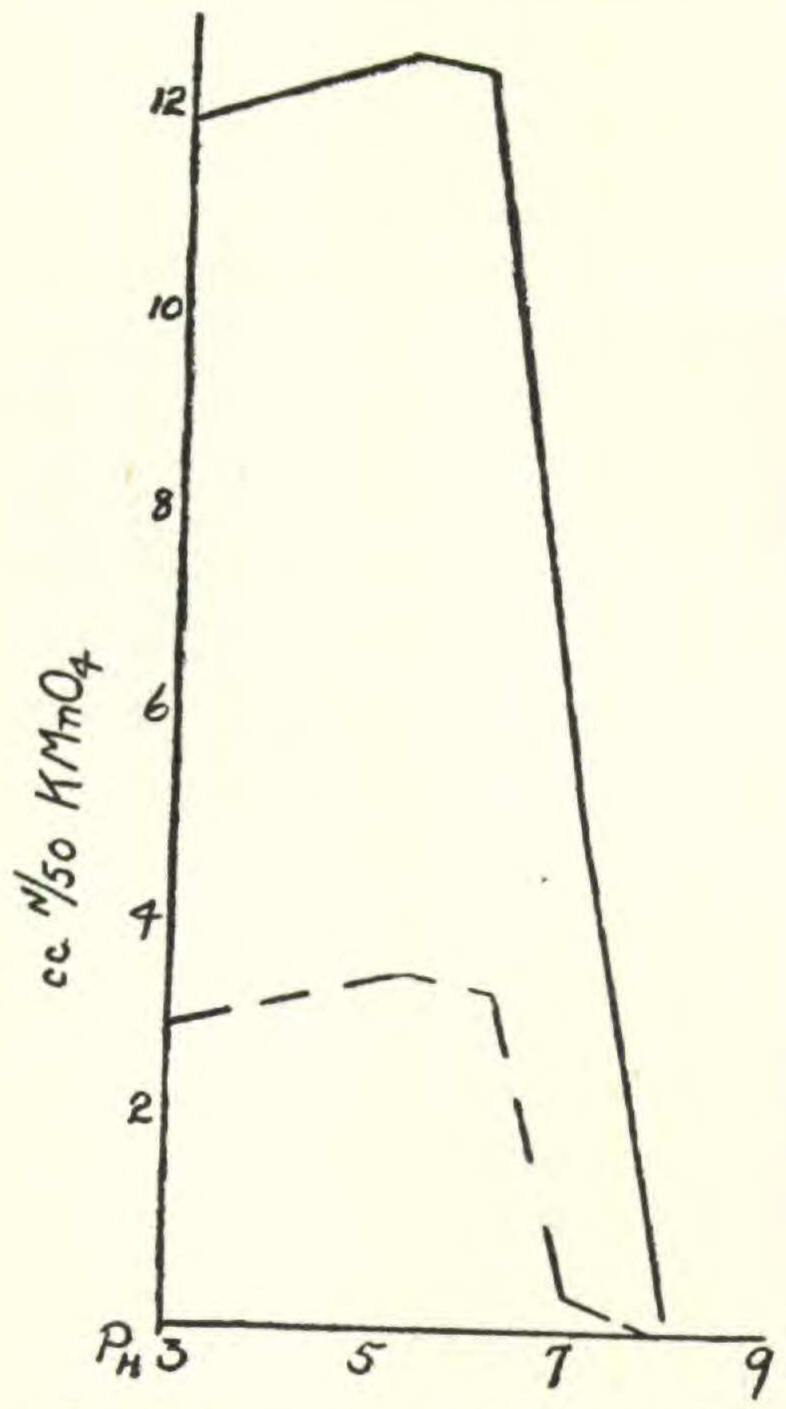

Fig. 10. Action of extracellular (——) and intracellular (- - -) amylase produced by *Penicillium italicum* grown in Czapek's solution of P_H 3.0.

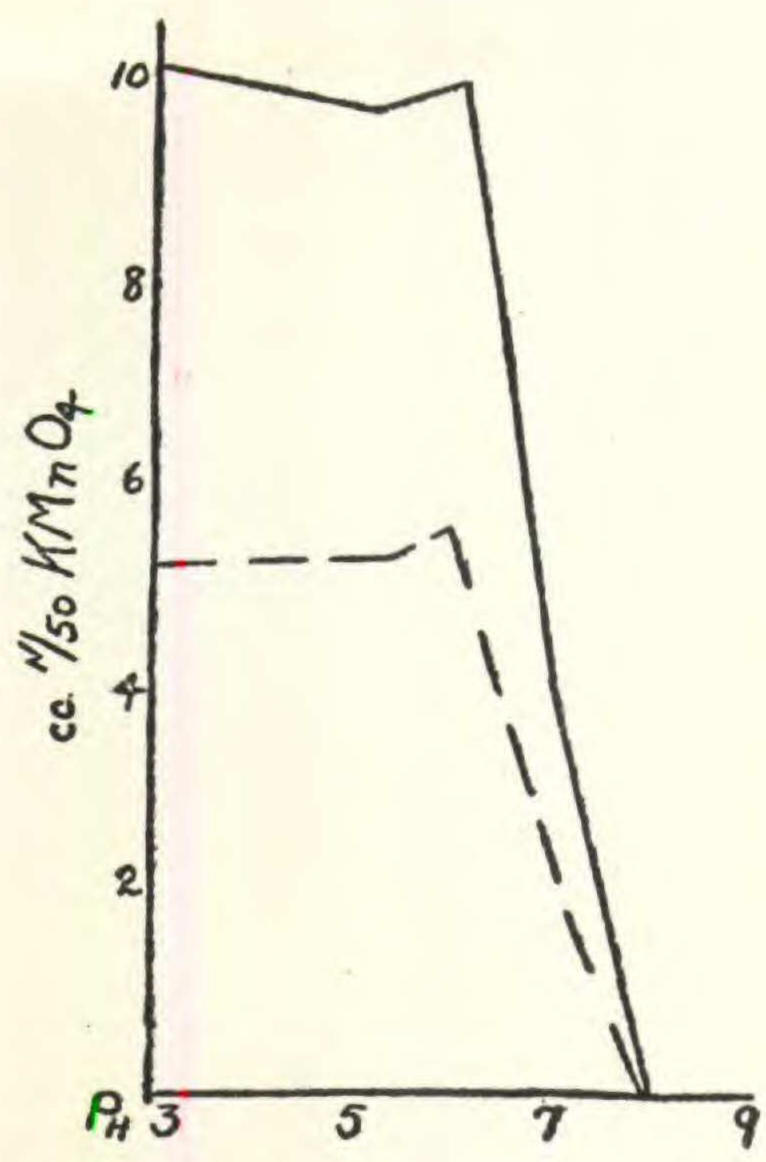

Fig. 11. Action of extracellular (——) and intracellular (- - -) amylase produced by *Penicillium italicum* grown in Czapek's solution of P_H 4.5.

slightly less in Czapek's solution having an H-ion concentration of P_H 3, and a gradual decrease occurred in Series 3, 4, and 5, respectively. The decrease on the alkaline side may have been due to several factors; either the alkalinity produced less secretion or the amylase was inactivated by these concentrations as it was produced. The amount of mycelium formed does not offer an explanation, since the dry weights of the fungous mats in the case of Series 5 were slightly more than in Series 1. How-

TABLE VI

RELATIVE TOTAL ACCUMULATION OF AMYLASE BY FUSARIUM SP., COLLETOTRICHUM GOSSYPII, AND PENICILLIUM ITALICUM WHEN GROWN IN CZAPEK'S SOLUTION OF VARIOUS H-ION CONCENTRATIONS

(A summation of tables II–IV inclusive)

Organism	Initial H-ion concentration of Czapek's solution									
	P_H 3.0		P_H 4.5		P_H 7.0		P_H 8.2		P_H 9.2	
	P_H of buffer sol.*	Cc. N/50 $KMnO_4$†	P_H of buffer sol.*	Cc. N/50 $KMnO_4$†	P_H of buffer sol.*	Cc. N/50 $KMnO_4$†	P_H of buffer sol.*	Cc. N/50 $KMnO_4$†	P_H of buffer sol.*	Cc. N/50 $KMnO_4$†
Fusarium sp.	3.0	8.2	3.0	7.0	3.0–3.2	1.0	3.1–3.2	1.6	3.0	2.3
	5.3–5.6	14.5	5.2	15.7	4.4–5.0	6.9	4.4–5.7	3.6	5.2–5.5	5.4
	6.0	16.9	6.0	18.7	6.0–6.8	7.3	6.1–6.2	5.9	6.0	5.9
	7.0	14.6	6.9–7.0	16.9	6.9–7.1	5.3	7.0	3.1	7.0	4.6
	7.7–8.0	9.8	7.5–8.0	10.5	7.6–8.0	2.2	8.0	0.4	8.0	3.0
	8.2–8.6	4.0	8.6–8.7	2.8	9.0	0.0	9.0	0.0	8.3–9.0	1.2
	11.0	0.4	11.0	0.0	11.0	0.0	11.0	0.0	11.0	0.0
Colletotrichum Gossypii			3.0	11.6	3.0	26.6	3.0	21.9	3.0–4.4	22.5
			5.2	17.6	5.1–5.5	27.8	5.2–5.6	25.5	5.4–6.2	25.9
			6.0–6.1	19.3	5.9–6.0	30.0	5.9–6.0	28.7	6.0–6.5	24.1
			7.0	17.8	7.0	26.3	6.9–7.0	21.7	6.9–7.2	18.7
			8.0–8.2	9.9	8.0	23.2	8.0	16.8	8.0–8.1	10.1
			8.4	4.2	8.5–8.8	7.2	9.0	9.0	8.4–9.1	4.2
			11.0	0.0	11.0	0.0	11.0	0.4	11.0	0.0
Penicillium italicum	3.0	15.0	3.0	15.4						
	5.1–5.2	16.2	5.1–5.4	15.1						
	6.0	15.9	6.0–6.1	15.7						
	7.0	5.8	7.0–7.1	6.2						
	8.0	0.2	8.0	0.0						
	9.0	0.0	9.0	0.0						
	11.0	0.0	11.0	0.0						

* H-ion concentration of buffer solution in which enzyme activity was measured.

† Amounts of sugar formed by extracellular amylase + intracellular amylase, as shown in tables II–IV inclusive.

ever, this variation was considered within the limits of experimental error, the weights produced being small. It has been shown in these results and also in the investigations of others that alkalinity produces inhibition of enzyme activity. This would explain the small curve obtained for the extracellular amylase. However, a similar reduction occurred for the intracellular enzyme which may mean that either the cell sap had alkaline properties similar to the culture solution and inhibited the enzyme as it was produced, that the culture solutions increased the permeability of the cells to the enzyme, or that the effect was upon the secretion itself.

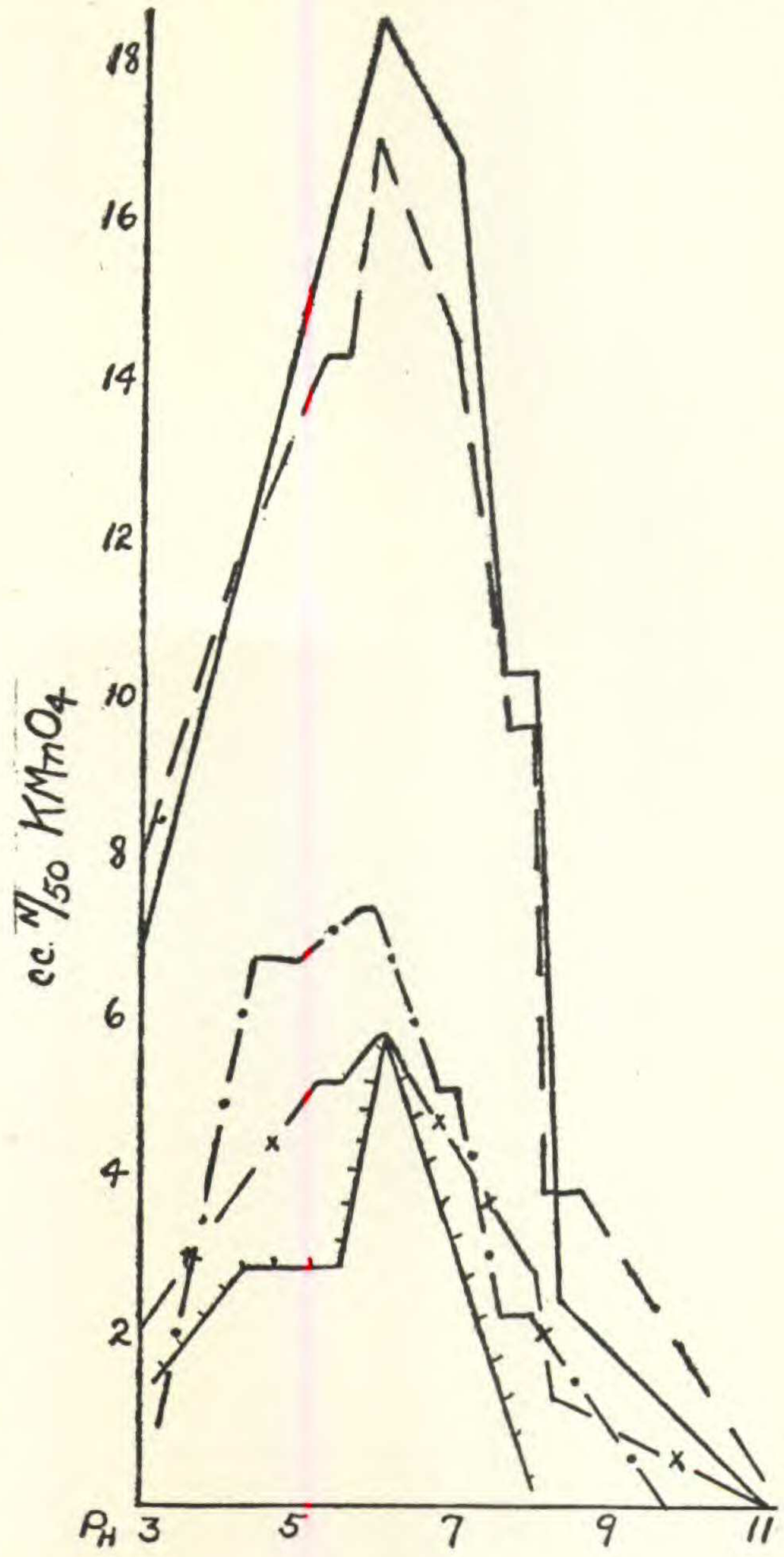

Fig. 12. Total action of extra- and intracellular amylase produced by *Fusarium* sp. grown in Czapek's solution of P_H3.0 (- - -), P_H4.5 (——), P_H7.0 (- · -), P_H8.2 (⊥⊥⊥), P_H9.2 (x—x).

The results obtained for *Colletotrichum* (fig. 13 and table VI) were quite different from the above. Accumulation seemed to increase as the cultures became less acid, which was evident in both the intra- and extracellular amylase determinations. Maximum accumulation occurred in the P_H 7 series but there was only slightly less in the P_H 8.2 series. At P_H 9.2 there was more activity than in any one of the series with *Penicillium* or with *Fusarium*. In a comparison of these organisms, it must be remembered that the reaction of the medium after the growth

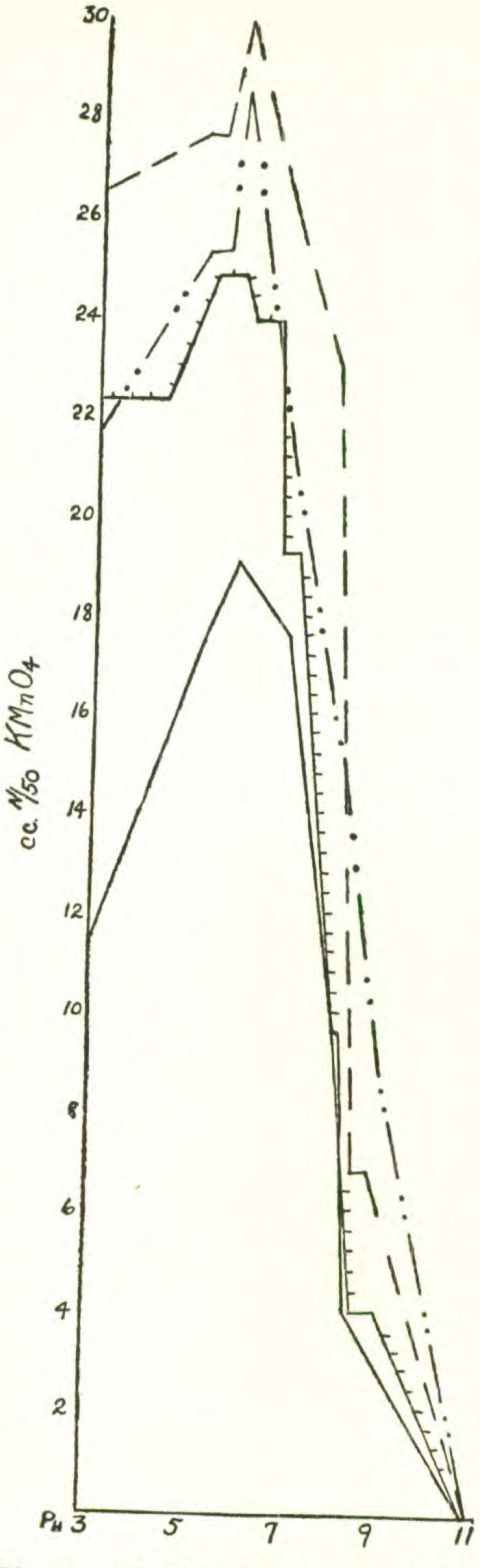

Fig. 13. Total action of extra- and intracellular amylase produced by *Colletotrichum Gossypii* grown in Czapek's solution of P_H 4.5 (——), P_H 7.0 (- - -), P_H 8.2 (· · — · ·), P_H 9.2 (┴┴┴┴┴┴).

of the fungus was not the same in the corresponding solutions. Thus for *Colletotrichum* it was P_H 8.4 in Series 8, while under similar initial conditions the final reaction in the case of *Fusarium* was P_H 9.

Sufficient data, in the experiments with *Penicillium*, have not been obtained to establish definite relations. However, natural Czapek's solution and Czapek's solution having a reaction of P_H 3 were equally effective in producing an accumulation of amylase (fig. 14 and table VI). In these determinations, it was evident that the amount of amylase excreted into the culture solution and the amount formed in the mycelium was greater than in the other organisms in the corresponding culture solutions.

A resume of the results with the three fungi showed that maximum amylase accumulation was effected by *Colletotrichum Gossypii* and the least by *Fusarium* sp. Although the results as yet are not inclusive enough to warrant absolute statements, yet they indicate that H-ion concentration of the culture solution may be a factor in amylase secretion. The H-ion concentration at which maximum accumulation was produced varied

with the organism. In *Colletotrichum* it occurred in the cultures having an initial H-ion concentration of P_H 7.0 and a final one of P_H 7.9, and in *Fusarium* in the cultures of P_H 3–4 which finally were P_H 7.2–7.8. The results with *Penicillium* did not cover conditions which were contrasting enough to furnish any conclusions.

Summary

The activity of amylase produced by fungi grown in Czapek's solution containing starch and having different degrees of acidity and alkalinity has been studied for *Fusarium* sp., *Colletotrichum Gossypii*, and *Penicillium italicum*. The activity of the enzyme as produced under these conditions was measured in $NaOH$-H_3PO_4 buffer solutions having H-ion concentrations from P_H 3.0 to P_H 11.0. An attempt has been made to determine the effect of the reaction of the culture solution upon the accumulation of amylase by the various organisms.

From the results obtained the following conclusions can be drawn:

1. A relation, which varies with the organism, seemed to exist between the H-ion concentration of the medium and the accumulation of extra- and intracellular amylase.

2. In *Fusarium* sp., maximum total accumulation was produced in the solutions having an initial of P_H 4.5 and a final reaction of P_H 7.8, whereas in *Colletotrichum Gossypii* a culture

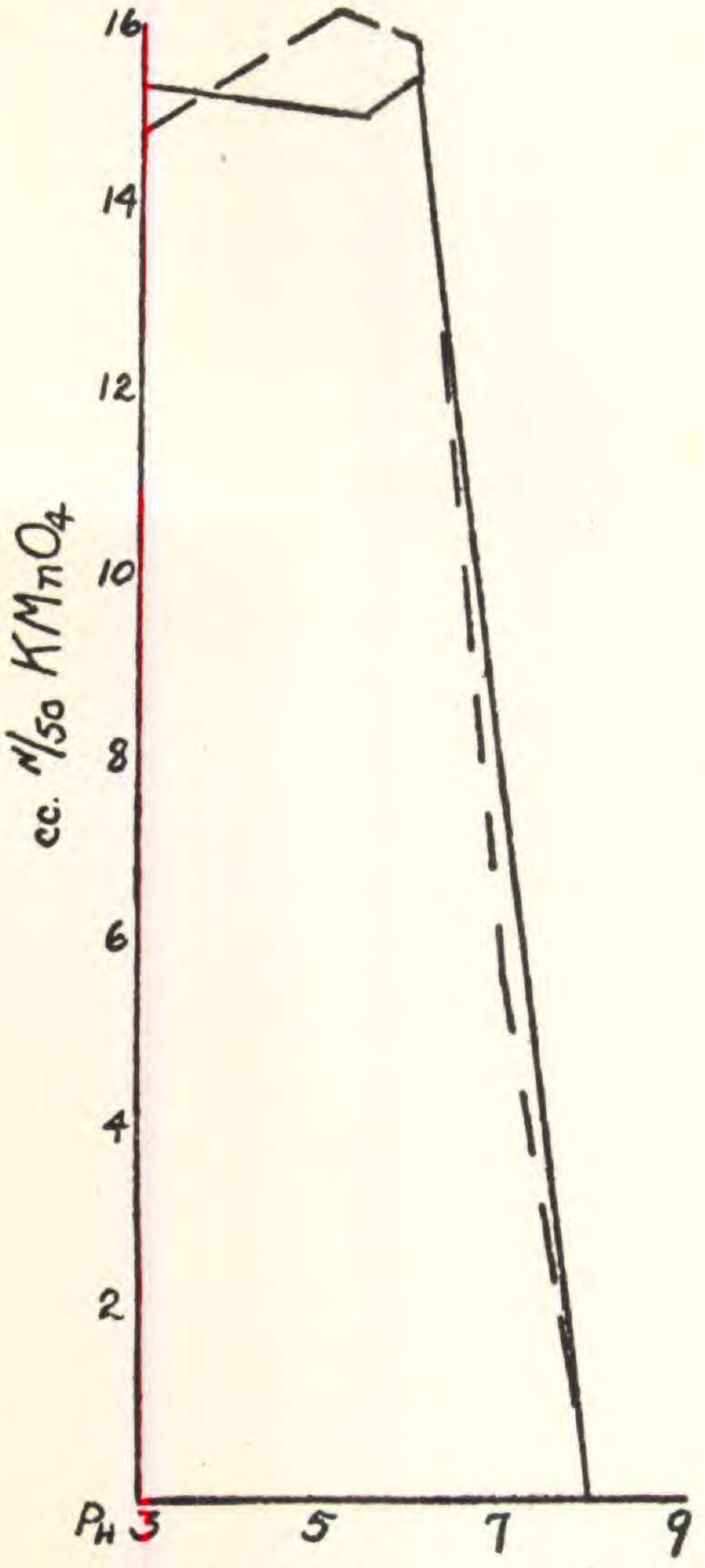

Fig. 14. Total action of extra- and intracellular amylase produced by *Penicillium italicum* grown in Czapek's solution of P_H 3.0 (- - -) and P_H 4.5 (——).

solution with an initial of P_H 7.0 and a final reaction of 7.9 afforded maximum results, but only slightly less accumulation occurred at P_H 8.2. Culture solutions of P_H 3.0 and P_H 4.5 were equally favorable in the case of *Penicillium italicum.*

3. Amylase accumulated more abundantly in the cultures of *C. Gossypii* than in the other fungi studied.

4. A gradual decrease in the amylase accumulation was effected by *Fusarium* as the culture solution became more alkaline, this decrease not being coincident with a reduction in the amount of growth.

5. An increase in accumulation occurred in the intra- and extracellular amylase of *C. Gossypii* as the nutrient solution became less acid, neutral or alkaline solutions being most effective.

6. The intra- and extracellular amylase, produced by any one fungus under varying H-ion concentrations of the culture solution, had similar properties with respect to the effect of the reaction of the NaOH-H_3PO_4 buffer solution upon activation.

7. An optimum zone of activity, between P_H 3.0 and P_H 6.0, existed for *P. italicum*, while in the other fungi the optimum was more sharply defined at P_H 6.0 when the activity was measured in the buffer solution at 28° C. for 24 hours.

8. Complete inactivation occurred at P_H 8.0 for the amylase of *P. italicum.* Under similar amounts of amylase accumulation by *Fusarium* and *C. Gossypii*, inactivation was effected by solutions of P_H 9.0 to 11.0.

9. A decrease in the actual acidity of the culture solution occurred in all of the series of *P. italicum* and all but the most alkaline, or P_H 9.2, series of *Fusarium* and *C. Gossypii.* The former produced no change in the reaction of this culture solution, while the latter caused a slight shift toward neutrality.

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Graduate Laboratory, Missouri Botanical Garden.

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